SUMMARY

The order Nidovirales comprises a monophyletic group of viruses with positive-stranded RNA genomes that are classified in the families *Arteriviridae*, *Coronaviridae*, *Mesoniviridae*, and *Roniviridae*. They share a conserved genome organization and a characteristic set of key replicative proteins. Although, in principle, this suggests a conserved replication mechanism, it is currently unclear how far exactly the resemblance extends on a more detailed level. This is foremost due to our poor understanding of the role of most viral proteins in the replication cycle. In addition, most of the knowledge that was obtained predominantly derives from studies of only few coronaviruses, the nidovirus subgroup with the largest known genome and therefore presumably employing the most complex replication strategy. In contrast, thus far only limited attention was given to the RNA replicating and processing enzymes of arteriviruses, and none at all to those of mesoni- and roniviruses, whose genome sizes are (much) smaller than those of coronaviruses. Given this disparity, it may be premature to assume that within this divergent group of viruses essential steps of the viral replication cycle, like for example RNA synthesis and mRNA 5′ end modification, strictly follow the same mechanistic pathways.

The work described in this thesis addresses some poorly or uncharacterized (domains of) nonstructural proteins (nsps) that are likely involved in one or multiple steps of RNA replication and/or transcription of the prototypic arterivirus equine arteritis virus (EAV). After a short introduction on the nidovirus replication cycle and our knowledge of the molecular details of the unusual transcription and mRNA processing mechanism (chapter 1), chapter 2 presents the crystal structure of the enzymatically active EAV helicase nsp10, which was obtained and analyzed in close collaboration with Chinese colleagues. Interestingly, a strong resemblance between this viral protein and the conserved cellular helicase Upf1, in particular with respect to their N-terminal zinc-binding domains, became obvious. Since this cellular helicase has been implicated in a number of eukaryotic post-transcriptional quality control mechanisms, a role for nsp10 and its nidovirus homologs in genome expansion is proposed. This and other potential functions of the nidovirus helicase in RNA replication, transcription, and translation, as well as virion biogenesis are further discussed in chapter 3, which presents a review of our current knowledge about nidovirus helicases. Special emphasis is placed on gaps that still remain, facts that cannot be easily reconciled with our current understanding of the nidovirus replication mechanisms, and questions that need to be addressed in future.

Chapters 4 and 5 focus on one of the central arterivirus replication proteins, nsp9, which harbors the RNA-dependent RNA polymerase (RdRp) domain. Chapter 4 describes a carefully controlled study to investigate different polymerase activities that nsp9 may have,

including a previously claimed primer-independent RdRp activity. Despite considerable efforts, involving experiments with different preparations of nsp9 and assays performed in the presence of putative polymerase co-factors, no *in vitro* activity was observed that could be clearly attributed to this protein. Moreover, circumstantial evidence suggested that the previously reported activity may have been caused by a contamination of the recombinant nsp9 preparation with the T7 RNA polymerase used to drive its expression in *E. coli*. In arteriviruses, the RdRp domain is located in the C-terminal two-thirds of nsp9. In chapter 5, it is now described for the first time that the RdRp domain is flanked at its N-terminus by another domain that is conserved in all nidoviruses. However, unlike the situation for the RdRp domain, no homologs of this domain have been found in other RNA viruses. This domain is thus proposed to be a second marker for the *Nidovirales* order, besides the N-terminal zinc-binding domain of the helicase subunit. Residues that are part of three conserved sequence motifs were without exception associated with a newly discovered nucleotidylation activity of recombinant nsp9. It is thus proposed that this activity could play a role in the modification of the 5′ end of viral RNAs through either RNA ligation, protein priming of RNA synthesis, or guanylyl transfer during mRNA capping. Further research is required to definitely tie nsp9 to one of these pathways. Nevertheless, alanine substitution of any of these conserved residues was either lethal to EAV and severe acute respiratory syndrome coronavirus (SARS-CoV) or severely crippled these viruses, eventually resulting in reversion of the mutation. These results thus demonstrate the essential nature of this domain for virus replication, whatever its exact function will turn out to be.

Two methyltransferase activities, commonly required for capping of mRNAs, were previously identified in two ORF1b-encoded coronavirus proteins, nsp14 and nsp16. While the former has no counterpart among the arterivirus nonstructural proteins, the latter and the arterivirus C-terminal subunit nsp12 occupy equivalent positions in the ORF1b-encoded part of the replicase although the two proteins share no detectable sequence similarity. It is thus a long standing question, how arteriviruses may catalyze the 5′ end modification of mRNAs, and we therefore performed a first characterization of the entirely uncharacterized EAV nsp12 subunit (chapter 6). Based on the genomic position of its coding sequence, sequence alignment, and secondary structure prediction, it is hypothesized that nsp12 might represent a unique arterivirus methyltransferase that has diverged from its homologs beyond sharing appreciated similarity. To test this hypothesis, recombinant nsp12 was expressed in and purified from *E. coli* and tested alone and in combination with potential co-factors for N7- and 2′-O-methyltransferase activity. Although positive controls represented by the SARS-CoV methyltransferases (nsp14 and the nsp10:nsp16 complex) demonstrated the functionality of the assay, no activity was detected for EAV nsp12. Guided by the sequence alignment, an extensive set of EAV

mutants was generated and characterized with respect to their plaque phenotype and progeny titer, as well as their protein expression. These reverse genetics experiments revealed a number of phenotypes ranging from wild-type-like via non-spreading to replication-incompetent, which indicated that nsp12 is essential for viral replication.

The above chapters describing biochemical properties of selected proteins may ultimately contribute to the identification of drug targets to combat nidovirus infections. In chapter 7 the prerequisites under which the marketing of such an antiviral drug would be economically viable are analyzed. This project was realized under guidance of several specialists of one of the industrial partners, Janssen Infectious Diseases, of the EUVIRNA consortium, the Marie Curie Initial Training Network to which my research project belonged. This study concludes that, at the moment, none of the circulating nidoviruses constitutes a sufficiently sized market to warrant the considerable investments required for drug development. The situation may be different if a new highly-pathogenic virus would emerge, as exemplified in 2002 by SARS-CoV or 2012 by MERS-CoV. In view of such threats, pre-pandemic drug stockpiling could be considered. However, also under those circumstances, it seems likely that the inherent financial risk would preclude an independent private initiative, even though market parameters and approval procedures appear to be favorable.

Finally, chapter 8 connects some of the main findings described in this thesis with previously described data. In particular, potential differences between small and large nidoviruses on the level of the molecular mechanisms of RNA synthesis initiation and mRNA capping are highlighted. To this end, alternative mechanisms are considered that would be consistent with the data on arteriviruses presented in this thesis and elsewhere. Furthermore, potential roles of cellular helicases in nidovirus replication and the host's immune response against nidoviruses are discussed.